

## **AMENDMENTS TO THE SPECIFICATION**

Please enter the enclosed sequence listing into the above-referenced application.

Please amend the paragraph beginning on line 3 of page 6 to read as follows:

Figure 1. (A) The mutacin III biosynthesis genes. The orientation of the genes and their relative sizes are shown. *mutA* is the structural gene for prepromutacin I, and *mutA'* has no known function at present. *mutB* and -*C* encode the enzymes for dehydration and thioether bridge formation of premutacin I. *mutD* encodes a flavoprotein possibly responsible for oxidative decarboxylation of the C-terminal cysteine in premutacin I. *mutP* and -*T* code for the protease and ABC transporter, respectively, which are responsible for the processing and transportation of premutacin I. (B) Similarity between MutA (SEQ ID No: 5) and MutA' (SEQ ID No: 6). The middle row shows the identical amino acids and the conserved changes (+). Arrowhead indicates the processing site in MutA (SEQ ID No: 5). The leader peptide and the mature peptide moieties were determined based on MutA (SEQ ID No: 5). (C) Effects of *mutA* and *mutA'* mutations on mutacin I production. Cells from an overnight culture plate were stabbed on TH agar plate and incubated at 37°C for twenty-four hours. The plate was heated at 80°C for one hour to kill the producing bacteria, then an overnight culture of the indicator strain NY101 was overlaid on top of the plate. The plate was inspected after an overnight incubation at 37°C.

Please amend the paragraph beginning on line 20 of page 6 to read as follows:

Figure 2. Similarity between the mutacin I (SEQ ID No: 5) and mutacin III (SEQ ID No: 18) structural gene. The prepropeptides of mutacin I (SEQ ID No: 5) and mutacin III (SEQ ID

No: 18) are compared using the sequence of preepidermin (SEQ ID No: 19) as a reference. The identical amino acids shared by all three lantibiotics are labeled with gray boxes, and the amino acids shared by any of two lantibiotics are labeled with an open box. The conserved sequence FNLD, which is shared by all lantibiotics in subgroup AI (29) is underlined. Brackets indicate the pairs of amino acid residues involved with thioether bridge formation in epidermin (1).

Please amend the paragraph beginning on line 7 of page 11 to read as follows:

The above discussion provides a factual basis for the preparation and use of mutacin I (SEQ ID No: 2). The methods used with and the utility of the present invention can be shown by the following non-restrictive examples and Figures.

Please amend the paragraph beginning on line 14 of page 12 to read as follows:

A therapeutically effective amount is an amount of mutacin I polypeptide (SEQ ID No: 2), the pharmaceutically acceptable salts, esters, amides, and prodrugs thereof, that when administered to a patient or subject, ameliorates a symptom of the condition or disorder.

Please amend the paragraph beginning on line 18 of page 22 to read as follows:

**Isolation and purification of mutacin I.** For mutacin production, CH43 was grown on TH/agar plate for one day under anaerobic conditions. The cells were then spread on a PHWP membrane with 0.3  $\mu$ m pore size (Millipore Corp., Bedford, MA) on top of a TH plate containing 0.3% agarose. The plate was incubated at 37°C for two days anaerobically. The membrane was transferred to a new plate for continued incubation every two days, and the old plate was frozen at -70°C. For mutacin isolation, the plates were thawed quickly in a 60°C water

bath. The liquid medium was separated from the agarose debris by centrifugation and the supernatant was passed through a membrane with 0.45 µm pore size. Mutacin I (SEQ ID No: 2) was extracted with an equal volume of chloroform. Novak et al. (1994) *J. Bacteriol.* **176**:4316-4320. The precipitate was dried under a stream of air and washed once with double-distilled H<sub>2</sub>O (ddH<sub>2</sub>O). The water-insoluble material (crude extract) was dissolved in 6 M urea and tested for antimicrobial activity by a plate assay after a serial dilution with ddH<sub>2</sub>O. One arbitrary unit of activity (AU) was defined as the highest dilution that showed a clear zone of inhibition of the indicator strain NY101.

Please amend the paragraph beginning on line 12 of page 23 to read as follows:

For purification, the crude extract of mutacin I (SEQ ID No: 2) was applied to a Source 15RPC column and eluted with a fragmented gradient A (0.1% TFA) and B (0.085% TFA in 60% acetonitrile) using a LKB Purifier (Amersham Pharmacia Biotech, Piscataway, NJ). The active fractions were pooled and dried in a lyophilizer. The pellet was redissolved in 0.25% TFA and subjected to a second round purification using a fragmented gradient of buffer A (0.1% TFA) and B (0.085% TFA in 80% methanol). The single active peak fraction was collected, dried in a lyophilizer, and used for sequence analysis and electrospray ionization mass spectrometry (EIMS).

Please amend the paragraph beginning on line 21 of page 23 to read as follows:

**Chemical modification of mutacin I.** Fifty micrograms of purified mutacin I (SEQ ID No: 2) were dried under vacuum and resuspended in 90 µl of a derivatization mixture consisting of 280 µl ethanol, 200 µl water, 65 µl 5M sodium hydroxide, and 60 µl ethanethiol as described).

Meyer et al. (1994) *Anal. Biochem.* **223**:185-190. The reaction proceeded at 50°C for one hour under nitrogen, then stopped by the addition of 2 µl acetic acid. The reaction mixture was dried under vacuum and washed three times with 50% ethanol. The pellet was resuspended in 10 µl of 50% acetonitrile with 1% formic acid for EIMS analysis and peptide sequencing by Edman degradation.

Please amend the paragraph beginning on line 14 of page 25 to read as follows:

**Similarity between mutacin I and mutacin III biosynthesis genes.** The overall similarity between mutacin I and mutacin III biosynthesis genes was ~94% at the nucleotide level over the 10 kb operon. However, the differences between the two operons were not distributed evenly among the different genes. For example, from *mutR* to the region immediately upstream of *mutA*, the similarity was 99%, while in the *mutA* and *mutA'* coding regions, the similarity was only 89% and 91%, respectively. At the amino acid level, the two MutAs (SEQ ID Nos: 5 and 18) shared 84% identical residues as shown in Figure 2, and the two MutA's shared 93% identical residues. For MutB and MutC the similarity was 93% and 95%, respectively. An even higher similarity (99%) existed in MutP and -T between the two strains.

Please amend the paragraph beginning on line 3 of page 26 to read as follows:

**Purification of mutacin I.** To biochemically characterize mutacin I (SEQ ID No: 2), sufficient amount of starting material is required. Applicants' first attempt to isolate mutacin I from liquid culture failed because no mutacin I was produced in any of the liquid cultures that were tested. A stab culture on TH/agarose plate as described for mutacin III was then tried. Qi et al. (1999) *Appl. Environ. Microbiol.* **65**:652-658. Mutacin I (SEQ ID No: 2) was produced on

such a plate, however the production level was still too low for satisfactory isolation. Based on the observation that mutacin I could be produced on all solid media plates regardless of the media composition, it was reasoned that the production of mutacin I may be regulated by a cell-density mediated control mechanism similar to quorum sensing. (Kleerebezem et al. (1997) *Mol. Microbiol.* **24**:895-904; Surette et al. (1999) *Proc. Natl. Acad. Sci. USA* **96**:1639-1644). Based on this rationale, a membrane transfer technique as described in Materials and Methods was employed, which resulted in a high level of mutacin I production.

Please amend the paragraph beginning on line 17 of page 26 to read as follows:

Mutacin I (SEQ ID No: 2) was purified by reverse-phase HPLC as shown in Figure 3. The active fraction (fraction 6) from the first pass (see Figure 3A) was collected and subjected to a second round purification using a different buffer B and a different gradient (see Figure 3B). The active fractions (fractions 6 and 7) from the second pass were dried under vacuum and tested for purity by EIMS analysis. As shown in Figure 3C, mutacin I was purified to near homogeneity as judged by the lack of significant background peaks in the MS chromatogram.

Please amend the paragraph beginning on line 3 of page 27 to read as follows:

**Characterization of mutacin I by ethanethiol derivatization and MS analyses.** The molecular weight of mutacin I (SEQ ID No: 2) was measured by electrospray ionization mass spectrometry. The mass-to-charge ratio for the doubly-charged molecule was 1183, and that for the triply-charged molecule was 788 as shown in Figure 3C. Thus the measured molecule mass was 2364 Da. This value was in a good agreement with the calculated value of 2516 Da for the

unmodified mutacin I minus six molecules of water (108 Da) and one molecule of carboxy residue (45 Da from decarboxylation at the C-terminal cysteine residue).

Please amend the paragraph beginning on line 11 of page 27 to read as follows:

The primary sequence of mutacin I (SEQ ID No: 2) contained six serine residues and one threonine residue, all of which were potential sites for post-translational dehydration. To confirm that there were indeed six dehydrated residues in the mature mutacin I, an ethanethiol modification of mutacin I under alkaline conditions was performed. In this reaction, one molecule of ethanethiol could insert into the thioether bridge, resulting in a S-ethylcystein and a cystein, or it could insert into the double bound of a dehydrated serine or threonine to form a S-ethylcystein or a  $\beta$ -methyl-S-ethylcysteine. Meyer et al. (1994) *Anal. Biochem.* **223**:185-190; Novak et al. (1996) *Anal. Biochem.* **236**:358-360. Ethanethiol derivatization of lantibiotics has been used prior to sequencing of the other lantibiotic gallidermin and pep5 (Meyer et al. (1994) *Anal. Biochem.* **223**:185-190), and for determination of the number of dehydrated amino acid residues in mutacin II (Novak et al. (1996) *Anal. Biochem.* **236**:358-360). The expected molecular mass of mutacin I after each addition of an ethanethiol molecule is listed in Table 1.

Please amend the paragraph beginning on line 13 of page 29 to read as follows:

To get a complete sequence of mutacin I (SEQ ID No: 2), the ethanethiol-derivatized mutacin I had to be used. Ethanethiol-derivatization of lantibiotics was shown to allow Edman degradation to proceed through the dehydrated serine and threonine residues and thioether bridges in other lantibiotics. Meyer et al. (1994) *Anal. Biochem.* **223**:185-190; Mota-Meira et al. (1997) *FEBS Lett.* **410**:275-279. Since the majority of mutacin I molecules was broken into two

fragments (see Figure 4) during ethanethiol modification, the C-terminal fragment had to be eliminated to solve the problem of having two N-termini in the reaction mixture. After several trials, the C-terminal fragment was eliminated by washing the reaction mixture with 30% acetonitrile. The pellet fraction after 30% acetonitrile wash contained mostly the full-length modified mutacin I and the N-terminal fragment. Sequencing of the pellet fraction revealed the following sequence: F<sub>1</sub>-SEC<sub>2</sub>-SEC<sub>3</sub>-L<sub>4</sub>-SEC<sub>5</sub>-L<sub>6</sub>-SEC<sub>7</sub>-SEC<sub>8</sub>-L<sub>9</sub>-G<sub>10</sub>-SEC<sub>11</sub>-T<sub>12</sub>-G<sub>13</sub>-V<sub>14</sub>-K<sub>15</sub>-N<sub>16</sub>-P<sub>17</sub>-SEC<sub>18</sub>-F<sub>19</sub>-N<sub>20</sub>-SEC<sub>21</sub>-Y<sub>22</sub>-SEC<sub>23</sub>. S-ethylcysteine (SEC) was the product of ethanethiol insertion into the double bond of dehydrated serine, or the thioether bridge in lanthionine. The results revealed that all six serine residues in the mutacin I molecule were dehydrated, and that T-12 remained as a nondehydrated residue. In addition, a closer look at the HPLC chromatogram of the sequencing reaction of mutacin I revealed minor peaks in the sequence of P-x-F-N-x-Y. This sequence correlated with the C-terminal fragment of mutacin I: P<sub>17</sub>-S<sub>18</sub>-F<sub>19</sub>-N<sub>20</sub>-S<sub>21</sub>-Y<sub>22</sub>-C<sub>23</sub>-C<sub>24</sub>. This result corroborated the previous assignment for the two peptide fragments generated during ethanethiol modification as shown in Figure 4B.

Please amend the paragraph beginning on line 5 of page 31 to read as follows:

A closer inspection of the differences between the homologous genes of mutacin I and mutacin III revealed that they are not all distributed evenly. For MutR, -D, -P, and -T, the homology is over 99% between the two mutacins, while for MutA, -A', -B, and -C, the similarity varies from 87 to 95%. The distribution of the variations within a protein is not even either. For example, in MutA (SEQ ID Nos: 5 and 18), the leader peptide region was identical between the two mutacins. However, the mature peptide region differed by 37.5% (Fig. 2). More interestingly, the sequence of the mature mutacin III is closer to that of epidermin (77%

similarity) than to mutacin I (62.5% similarity), while the sequence of the leader peptide of mutacin III and epidermin are dramatically different as seen in Figure 2. For MutB, -C, -D, -P, and -T proteins, mutacin I and mutacin III are closer to each other than to epidermin.

Please amend the paragraph beginning on line 9 of page 32 to read as follows:

Mutacin I and mutacin III are closely related to each other at both the nucleotide and amino acid levels. Comparison of the mature peptide sequence of mutacin I and mutacin III suggests that they may also have the same pattern of thioether bridge formation. Despite all the similarities, some important differences exist between the two mutacins. For example, ethanethiol modification of mutacin I broke the molecule into two fragments between N-16 and P-17 as shown in Figure 4B, while the same reaction did not affect the integrity of mutacin III as shown in Figure 4C. Comparison of the two mutacins (SEQ ID Nos: 5 and 18) revealed that the major difference is at the linker region (T-12 to P-17), where mutacin I has the sequence T-G-V-K-N-P, and mutacin III has the sequence A-R-T-G as shown in Figure 2A. These different amino acid residues, according to the statistical figures of Creighton (Creighton, p. 235, in (ed.) Proteins: Structures and molecular principles, W.H. Freeman and Company, New York), have different tendencies in forming different secondary structures in proteins. For example, N-16 and P-17 in mutacin I are more likely to be involved in forming  $\beta$ -turns, while A-12 in mutacin III is more likely to participate in  $\alpha$ -helix formation (Stryer, p. 37, in (ed.) Biochemistry, W.H. Freeman and Company, Biochemistry, New York). More importantly, N-16 and P-17 are absent in mutacin III.